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A competitive dual-label time-resolved fluoroimmunoassay for the simultaneous determination of chloramphenicol and ractopamine in swine tissue

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A novel dual-label time-resolved fluoroimmunoassay method was developed for the simultaneous determination of chloramphenicol (CAP) and ractopamine (RAC) residues in 18 swine tissue samples, using anti-CAP and anti-RAC monoclonal antibodies labeled with europium (Eu^{3+}) and samarium (Sm^{3+}), respectively. The detection limits for CAP and RAC were 0.06 and 0.25 ng/g. The recovery from swine muscle samples was 102%–121% for CAP at spiking levels of 0.1–5 ng/g, and 69.8%–85.8% for RAC at spiking levels of 1–10 ng/g. The results obtained from the swine tissue samples using this method showed good agreement with those obtained using ELISA and GC-MS, with correlation coefficients (R) between 0.92–0.98.

dual-label time-resolved fluoroimmunoassay, chloramphenicol, ractopamine, residue, swine tissue

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Chloramphenicol (CAP) is a broad spectrum antibiotic often used in veterinary practice for the prevention and treatment of many bacterial diseases. However, continual use in pigs may result in a build-up of CAP residues in tissue, and this may have toxic effects on consumers [1,2]. Therefore, the administration of CAP has been banned in many countries including the EU, the USA and China. Techniques such as liquid chromatography (LC) [3], liquid chromatography-mass spectrometry (LC-MS) [4], LC-MS/MS [5,6], and others [7–10] are currently used to determine CAP levels.

Ractopamine (RAC) is a β_2-adrenoceptor agonist approved for use in finishing swine to improve weight gain, lean muscle mass and feed efficiency [11]. It is not permitted for use in food-producing animals in the EU and China because of the potential danger to humans [12,13]. Therefore, developing sensitive analytical methods for monitoring the levels of RAC residues in animal-derived food stuffs is of great importance. The currently available methods for RAC determination are mainly based on LC-MS [14–16].

Because CAP and RAC are strictly controlled and widely tested for animal-derived foods, it is of interest to develop methods for the simultaneous determination of these two analytes. To date, most multi-analyte assays for veterinary drug residues have focused on structurally related compounds [17–22], and no method has been developed for the simultaneous detection of different drug classes in animal tissue samples.

In this study, a novel dual-label time-resolved fluoroimmunoassay (TRFIA) assay was developed for the simultaneous detection of CAP and RAC in swine tissue using anti-CAP and anti-RAC monoclonal antibodies (Mabs) labeled with europium (Eu^{3+}) and samarium (Sm^{3+}), respectively. After reaction with the labeled Mabs [23,24] and treatment with co-enhancement solution, CAP and RAC in the samples were detected by measuring the fluorescence emissions of Eu^{3+} and Sm^{3+}, respectively.

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1 Materials and methods

1.1 Reagents and materials

A Mab against CAP was produced according to the method of Kolosova [25], and showed insignificant cross-reactivity with other fenicol drugs such as thiamphenicol and florfenicol [10]. A Mab against RAC was obtained from the college of veterinary medicine at China Agricultural University [26]. DTTA-Eu^{3+} and DTTA-Sm^{3+} were purchased from Tianjin Radio-Medical Institute (Tianjin, China). CAP, RAC, ovalbumin (OVA), and bovine serum albumin (BSA) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Transparent 96-well micro-titration strips were purchased from Nunc (Roskilde, Denmark). Sephadex 6B and G-50 were purchased from Pharmacia (Uppsala, Sweden). Commercial ELISA kits for determining RAC and CAP residues in animal tissue were purchased from Wanger Biology Co. Ltd (Beijing, China). Other reagents were of analytical quality and supplied by Beijing Reagent Corporation (Beijing, China).

1.2 Labeling of Mab with europium and samarium chelate

The Mabs against CAP and RAC were dialyzed twice in coating buffer (0.1 mol/L carbonate containing 0.05% NaN_3 and 0.9% NaCl, pH 7.8) for 24 h, then mixed with 1 mg DTTA-Eu^{3+} or DTTA-Sm^{3+}, respectively, in small brown bottles. The resulting Mab-DTTA-Eu^{3+} and MAb-DTTA-Sm^{3+} chelates were purified by gel filtration on a Sephadex 6B/G-50 column (50 cm × 1.5 cm, Pharmacia, Uppsala, Sweden) and eluted using washing buffer (0.05 mol/L Tris-HCl, pH 8.0, containing 0.9% NaCl and 0.04% Tween 20). Because of their high molecular weight, the chelates were collected before any dissociated Eu^{3+} and Sm^{3+}. After adding 0.1% BSA and 0.04% NaN_3, part of the purified chelate solution could be stored at –20°C for up to a year. Those used in this study were maintained at 4°C. The molar ratio of labeled $Eu^{3+}$and Sm^{3+} to Mab was determined by fluorescence measurement at 280 nm (A_{280}) and calculated using the equation: $Y = C([Eu^{3+}], \mu mol/L)/C(Mab, \mu mol/L)$.

1.3 Time-resolved fluoroimmunoassay procedure

The coating antigens (OVA-CAP and OVA-RAC) were mixed with coating buffer, pipetted into microtiter plate strips (Nunc, Roskilde, Denmark) and incubated for 14 h at room temperature. After washing at least 3 times with washing buffer, the strips were blocked with blocking buffer (0.05 mol/L Tris-HCl, containing 0.5% BSA, 0.9% NaCl and 0.04% NaN_3, pH 8.0) at 37°C for 1 h. Test samples, or a series of standards, were then added to the wells along with the Eu^{3+}-labeled or Sm^{3+}-labeled Mabs diluted at the appropriate concentrations in dilution buffer (0.05 mol/L Tris-HCl containing 0.9% NaCl, pH 8.0). Finally, the co-enhancement solution (0.1 mol/L potassium biphthalate-acetic acid buffer containing 15 μmol/L β-naphthoyltrifluoroacetone and 0.1% Triton X-100) was added to each well, and the fluorescence signal measured using a VICTOR^2 multilabel counter (Perkin-Elmer Wallac, Turku, Finland). The fluorescence of Eu^{3+} was measured at an excitation wavelength of 340 nm and an emission wavelength of 615 nm, with a delay time of 0.40 ms and a cycle period of 1.0 ms. Sm^{3+} fluorescence was measured at an excitation wavelength of 340 nm and an emission wavelength of 642 nm, with a delay time of 0.05 ms and a cycle period of 0.10 ms.

1.4 Standard curve

A series of mixed standards with concentrations (CAP/RAC) of 0/0, 0.05/0.25, 0.1/0.5, 0.25/1.25, 0.5/2.5, 1/5, 2.5/12.5, 5/25, 10/50, 20/100 ng/mL were prepared by diluting the CAP and RAC standards in dilution buffer. Standard curves were obtained by plotting the fluorescence intensity (Y) against the logarithm of the sample concentration (X) and fitted to a four-parameter logistic equation using Origin Pro7.0 (Version 7.0, Microcal, USA):

$$Y=\{(A-D)/[1+(X/C)^B]\}+D, \quad (1)$$

in which A is the asymptotic maximum (fluorescence intensity in the absence of analytics, S_{max}), B is the curve slope at the inflection point, C is the X value at the inflection point (corresponding to the sample concentration that reduces S_{max} to 50%, IC_{50}), and D is the asymptotic minimum (background signal).

1.5 Sample pretreatment

Five grams of homogenized swine muscle was mixed with 15 mL of a solution containing acetonitrile and 0.01 mol/L HCl (84/16, *V*/*V*), vortexed for 1 min and centrifuged for 10 min at 5000×*g*. Three milliliters of the resulting supernatant was mixed with 2 mL of sodium borate buffer (pH 10.3) using a vortex. The mixture was then mixed with acetic ether and allowed to stand for 10 min. The upper fraction was then separated, evaporated to dryness, and the residue dissolved in 1 mL of dilution buffer before being defatted using n-hexane. After centrifuging for 10 min at 8000×*g*, the lower fraction (100 μL) was pipetted into the wells for analysis.

1.6 Validation using other methods

The new experimental method was verified using ELISA and GC-MS. ELISAs were conducted using commercially available ELISA kits (Wanger biology Co. Ltd, China) as previously described [14]. GC-MS was performed as previously described [27] using an Agilent (Palo Alto, CA, USA) model 6890 GC, a model 5973 mass-selective detector, and an HP-5 capillary column (0.25 mm×30 m×0.25 μm).

2 Results and discussion

2.1 Optimization of detection conditions

TRFIA performance depends on several experimental factors, including a suitable molar ratio of the labeled Eu^{3+} or Sm^{3+} to Mab, appropriate ratios of antigen and tracer (labeled Mab) and the addition ratio (V_{tracer}/V_{sample}), the buffer species used, and the immunoassay reaction time. These parameters were optimized for both CAP and RAC using standard mixtures, and S_{max}/IC_{50} as the major parameter to estimate the sensitivity. The results showed that the labeling ratio of Eu^{3+} : anti-CAP Mab was 4/1, and Sm^{3+} : anti-RAC Mab was 6/1. The greatest sensitivity was obtained using a tracer dilution (volume of labeled Mab:volume of dilution buffer) of 1/200 for CAP and 1/40 for RAC, an addition ratio of 100/100, antigen dilution ratios (volume of antigens : volume of coating buffer) of 6.7/20000 for RAC-ovalbumin and 45/20000 for CAP-ovalbumin, and an incubation time of 1 h.

It is generally believed that the greater the number of Eu^{3+} or Sm^{3+} bound to each Mab, the stronger the fluorescence signal obtained. However, we found that the immunoreactivity and stability of the bound chelate decreased at labeling ratios (Eu^{3+}:Mab and Sm^{3+}:Mab) higher than 20. The greatest sensitivity was obtained with labeling ratios between 4 and 10. Probably it is because higher ratios may weaken the affinity of the Mabs for the haptens. We also found that the fluorescence signals obtained after dual-labeling or single-labeling were not significantly different, suggesting that the dual-labeled method can be used to determine CAP and RAC levels without sacrificing sensitivity.

2.2 Analytical performance

Figure 1 shows the standard curves obtained from 10 separate assays. The limits of detection (LOD) that gave a 10% inhibition of the maximal Eu^{3+} and Sm^{3+} fluorescence were 0.06 ng/g for CAP and 0.25 ng/g for RAC. Muscle samples lacking the analytes were spiked with 0.1/1, 1/5, 5/10 ng/g of CAP/RAC, before analysis. The results, shown in Table 1, indicated that the recovery ranged from 102%–121% for CAP and 69.8%–85.8% for RAC. The intra-assay variation was 12%–17% for CAP and 10%–16% for RAC, while the inter-assay variation was 17%–20% for CAP and 17%–21% for RAC. These values are acceptable for the detection of CAP and RAC residues.

To further evaluate the applicability of the proposed method, 18 swine muscle samples were spiked with random concentrations of CAP and RAC and measured using ELISA, GC-MS and TRFIA. As shown in Figure 2, the results of TRFIA showed good agreement with those obtained using ELISA and GC-MS, with correlation coefficients (R) in the range of 0.92–0.98. Thus, dual-lable TRFIA is a reliable method for the simultaneous determination of CAP and RAC residues in swine tissue.

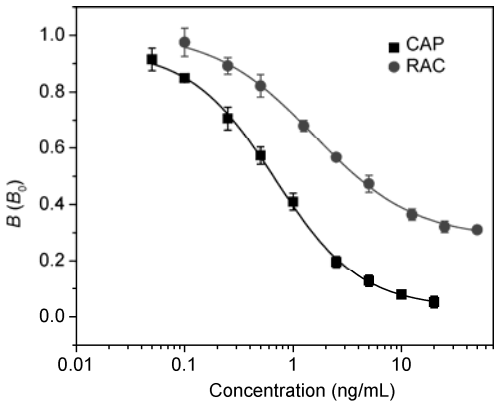

Figure 1 Typical standard curves for CAP and RAC. Each point represents the mean ± standard deviation of three replicates.

Table 1 Recovery of CAP and RAC from fortified blank swine muscle samples

Analyte	Added (ng/g)	Recovery (%, n=4)	Intra-assay (CV%, n=4)	Inter-assay (CV%, n=3)
CAP	0.1	121	17	20
	1	115	16	17
	5	102	12	18
RAC	1	69.8	14	21
	5	74.6	16	19
	10	85.8	10	17

3 Conclusion

In this study, we established a simple and sensitive TRFIA method for the simultaneous determination of CAP and RAC levels in swine tissue. This is the first report of a TRFIA method that can be used to for the simultaneous detection of drug residues belonging to different chemical classes. This novel procedure yielded significant reductions in assay time, and showed good correlation with ELISA and GC-MS. It is expected that this approach will allow the rapid detection and measurement of different classes of chemicals in animal tissue.

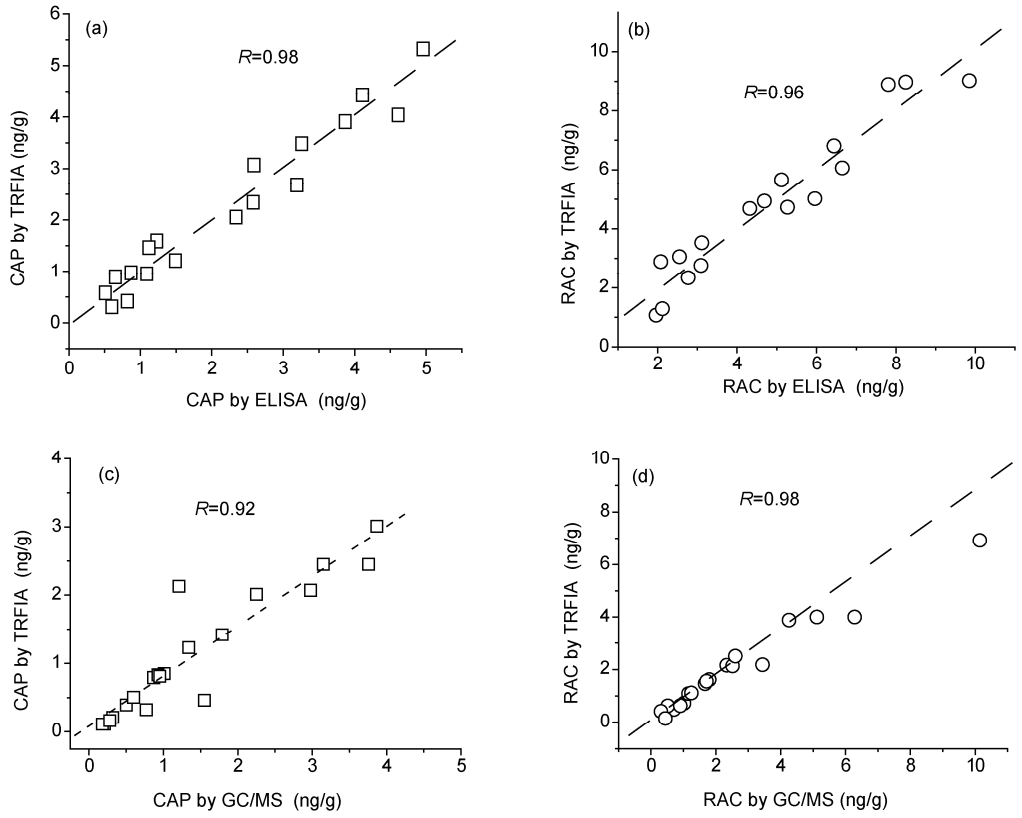

Figure 2 Correlation between TRFIA and ELISA ((a),(b)) and GC-MS ((c),(d)). □, concentration of CAP; ○, concentration of RAC.

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